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NUTRITION REVIEWS

VOL. 12 DECEMBER 1954 No. 12

EARLY EXPERIENCES WITH IRON AND COPPER—A RETROSPECT

In the fall of 1924 the late Professor E. B. Hart at the University of Wisconsin initiated a comprehensive study of the metabolism of iron and the nutritional factors influencing hemoglobin formation. The choice of the problem reflected Professor Hart's interest in mineral metabolism, particularly that of iron, and also in the nutritive properties of milk. As the problem moved along different aspects of it were undertaken, but the main effort involved the study of the development of an anemia in young animals restricted to a diet of milk and the prevention and cure of this anemia by various supplements. The results of these investigations appeared in a succession of papers during the following five years in the *Journal of Biological Chemistry* under the main title of Iron in Nutrition.

The first experiments utilizing young weaned rabbits maintained on a diet of cow's milk were carried out by C. A. Elvehjem and myself. We were both graduate students in the department and Elvehjem also had an appointment as an assistant. Our cooperative efforts on this problem, each being concerned particularly with certain phases of the work, were to continue until the study was practically at an end. The choice of the rabbit as the experimental animal seems, in retrospect, to have been mainly one of availability. Earlier that year I had started a rabbit colony in the department and had made some limited observations as to the utility of this species for nutritional investigations. A number of young weaned animals, and cages to house them, were available so it was decided to see if they would become anemic when made to subsist on a diet of milk. The early experiments established that the young rabbit did become anemic under those conditions and that the addition to the diet of inorganic iron, in the form of ferric oxide, did not prevent the anemia. On the other hand, the addition of a small supplement of fresh or dried cabbage or lettuce to the milk and iron diet prevented or cured the anemia rather promptly.

These findings were in agreement with what earlier workers in this field, such as von Bunge and his students, particularly E. Abderhalden, had reported. They had speculated that either an organic iron compound or some organic moiety in addition to iron was needed to permit the animal to synthesize hemoglobin. This latter theory seemed to obtain some support by our further findings that alcoholic extracts of dried cabbage, yellow corn, and even a preparation of chlorophyll seemed to increase the hemoglobin and the number of red cells in the blood of the young rabbits receiving a diet of milk and inorganic iron. The extracts were either free from iron or extremely low in iron and, therefore, seemed to contain some organic moiety of value. I recall that there was speculation that it might be a pyrrole-containing complex.

It should be mentioned that R. C. Herrin (now Professor of Physiology at the University of Wisconsin), another graduate student, joined us during the early stages of this problem, studying especially the effect of diet, particularly an iron supplement, on the quality of the milk produced by cows or by goats. It was found that diet had little or no effect on the iron content of the milk or on its ability to produce anemia in young rabbits.

The above is a brief summary of the way the problem moved forward during the first year and a half. In retrospect our progress was slow because we found the young rabbit to be something less than wholly satisfactory as a test animal. It

responded slowly to the different experimental regimens; even on the basal diet of milk and iron it continued to grow at a slow rate. Many of our animals were continued on experiment for many months and became quite large. Thus, we were limited in the number of animals we could observe. It was felt necessary to make both hemoglobin determinations and red cell counts to assess the degree of anemia. In contrast with what we did later with young rats, this part of the work was definitely less sure and precise and more laborious.

In early 1926 a significant advance was made when it was found that the ash of dried lettuce or of dried cabbage was equally as effective as the nonashed material in curing the nutritional anemia. This demonstration that the antianemia factor which we were studying was inorganic in nature was of great importance for its effect on our thinking and our plans for further work on this problem. I recall quite clearly the situation as it existed that spring because soon afterwards I had to start writing my thesis preparatory to receiving the Ph.D. in June.

In addition to receiving my degree, I also received an appointment as research associate in the department. The further effort which we were to put on the anemia problem and the manner in which it was to be studied had been matters for discussion in our group. Among other things, it was decided that we should attempt to use the young rat as the test animal. At this time, Professor Harry Steenbock became more closely associated with the problem and took a more active part in its direction. Also, in our further work we were assisted, first, by Blanche Riising and, later, by Evelyn Van Donk.

We were fortunate in finding the young growing rat a very useful test animal. Rather in contrast with what others had reported in the literature, our weanling rats, restricted to a milk diet, developed a profound anemia in a few weeks and proved to be highly sensitive in their response to potent supplements. Because of the marked anemia and the equally marked response we were able to rely on hemoglobin determinations and to dispense with the time-consuming red cell counts. With the greater speed and precision of our work and with the larger number of animals we were able to maintain on experiment, our progress was much greater than it had been during the previous two years.

In our first experimental work with anemic rats it was necessary to check some of our previous findings; in particular, to assess the value of different inorganic iron salts. Our use of the insoluble ferric oxide in our earlier experiments had been criticized by H. S. Mitchell and L. Schmidt (*J. Biol. Chem.* **70,** *471 (1926)*) who had found certain soluble iron salts to be effective in curing anemia in their animals. To throw light on this problem, I recall that we prepared from the same "pure" iron wire five different salts—chloride, sulfate, acetate, citrate and phosphate—using the purest reagents available in the laboratory. We found no difference between these preparations which was in any way related to their different solubilities. All of them were quite ineffective in curing the anemia when used at the dosage levels normally fed; some, however, when fed at very high levels brought about a small but measurable hemoglobin response. This latter situation was a puzzle to us at the time.

In other experiments we tested small supplements of a number of food products, such as dried liver, dried kidney, dried muscle tissue, wheat and corn. Some of these materials were also tested in the form of the ashed residues in comparison with lettuce ash which we had previously found to be effective. It was found that certain of these substances were more effective in curing the anemia than others, but it was also found by analysis that the supplements varied in iron content. When they were retested on the basis of equal iron intake there were measurable differences between

them (liver was pre-eminent), but what impressed us most was the great efficacy of very small doses of iron when offered in the form of these food materials (or their ashes) as compared with much greater doses in the form of the inorganic iron salts. By the time we had completed these studies we were all convinced that we were dealing with some impurity, inorganic in nature, which was needed in addition to iron for the formation of hemoglobin.

We had found the ash residues from some of the food products difficult to recover and to administer quantitatively. For example, the ash of beef liver tended to fuse to a glass-like residue which adhered to the crucible. In the thought of obtaining a more homogeneous product we digested the ash residues with hydrochloric acid and then filtered off the insoluble material. We were pleased to find that these acid extracts of the ash were equally as effective as the ash itself or as the original food as nearly as we could determine. With the knowledge that the active material could be obtained in solution in hydrochloric acid it was not difficult to decide to undertake fractionation experiments.

While preliminary fractionation experiments were carried out on lettuce ash, it is interesting to recall that our most successful separation was made using as starting material the ash of a commercial liver fraction prepared for the treatment of pernicious anemia. We had been fortunate to obtain from Eli Lilly & Company a generous sample of their first production of liver extract made according to the procedure of E. J. Cohn and his associates at Harvard (*J. Biol. Chem.* **74,** *lxix (1927)*). We had found the material to be an effective supplement for our anemic rats, particularly when given with a small addition of iron since it contained only 0.008 per cent iron. In fractionating the hydrochloric acid extract of the ash of this liver preparation we first treated it with hydrogen sulfide and obtained a small but perceptible precipitate which was filtered off. The filtrate was then treated with ammonia and ammonium sulfide to obtain a second precipitate. These two precipitates and the filtrate, when each was suitably prepared as a solution for feeding, were given to anemic rats in the presence of a supplement of inorganic iron. There was a marked difference in the therapeutic effect of the three supplements; only the fraction precipitated by hydrogen sulfide was active, the other two failing completely to increase the hemoglobin level of the anemic animals.

With the first inkling as to the nature of the "unidentified factor" and while the above experiments were still in progress, we decided to test the efficacy of copper as a supplement to iron in the cure of the nutritional anemia. We had often noted a pale blue color in some of the ash samples which we studied and this also had suggested copper salts. Our first trial was with a single anemic rat to whose diet of whole milk and ferric chloride we added a supplement of copper sulfate. The hemoglobin response was as prompt and pronounced as any we had previously noted with potent supplements. Additional tests with copper quickly followed, all of which demonstrated marked stimulation of hemoglobin synthesis when given with iron on the milk diet. Amounts of copper as little as 1 microgram per rat per day gave a measurable response in most cases, although amounts of the order of 25 micrograms per day were needed for a maximum effect.

It now became possible to understand many of the puzzling results of the past, not only our own but those of others. For example, copper was found to be a contaminant, in some degree, of practically all laboratory iron preparations, which explained the slow response of some animals to high doses of certain solutions of iron salts. When these solutions were treated to remove copper they no longer stimulated hemoglobin production. It was possible also to understand that certain samples of market milk, processed in copper equip-

ment, contained sufficient copper to interfere with the development of anemia in young animals. The presence of trace amounts of copper in practically all natural food products, especially animal products such as liver, had been pointed out by workers like J. S. McHargue (*Am. J. Physiol.* **72,** *583 (1925)*).

During the following year we carried out additional studies to see if any other element besides copper would function as a supplement to iron in curing the nutritional anemia produced by the milk diet. We included salts of such elements as zinc, cobalt, nickel, germanium, arsenic, manganese, and several others and found that none of these, alone or in combination, could replace copper.

In recalling these experiences it has always seemed to me that an important element of our success was the development of a precise animal test—the *sine qua non* of progress in all nutritional studies. I suspect also that we were fortunate in having a special supply of milk; it was delivered fresh every day direct from the University dairy barn without processing in any equipment. By comparison with some of the separations and identifications of nutritional entities since that time the feat of identifying copper as a necessary nutrient seems rather simple. It was, however, an important advance, perhaps as much for what it presaged in nutritional progress as for any other reason.

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